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THE EFFECTS OF CONTINUOUS AQUEOUS
EXPOSURE TO 14C-78.02 ON HATCHABILITY
OF EGGS AND GROWTH AND SURVIVAL OF
FRY OF FATHEAD MINNOW (Pimephales
promelas).

FC-95

RESEARCH REPORT

SUBMITTED TO

3M COMPANY

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ABSTRACT

Fathead minnow (Pimephales promelas) eggs and fry were continuously exposed to measured ^{14}C -labelled 78.02 concentrations ranging from 1.9 mg/l to 0.12 mg/l. Data were collected on percentage hatch of eggs and survival, total length and wet weight of fry after 30 days exposure.

Survival was the most sensitive indicator of ^{14}C -78.02 toxicity, while hatchability and growth were not affected. Based on the reduced survival of fish at 30 days post-hatch, the MTC value is estimated to be >1.0 mg/l and <1.9 mg/l ^{14}C -78.02.

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SECTION I

INTRODUCTION

The objective of this study was to determine the effects of ^{14}C -78.02 on fathead minnow (Pimephales promelas) eggs and fry during continuous aqueous exposure. Exposures were initiated within 48-hours after egg fertilization and continued through 30 days post-hatch. The effects on egg hatchability and on survival and growth of fry were measured and used to make an estimate of the MTC (minimum threshold concentration). The MTC is virtually synonymous with the term MATC (maximum acceptable toxicant concentration) developed by Mount and Stephan (1967). Mount and Stephan's term, however, was estimated after the performance of a full, life-cycle, chronic test where effects on reproduction and second generation fry were also measured.

Macek and Sleight (1977) and McKim (1977) described egg and fry investigations as being reasonably accurate short-term estimations of potential long-term chemical hazards to fish, and as being similar to those estimations derived from definitive chronic toxicity studies. In the majority of the studies reported by the authors and of those performed at this laboratory, the embryos and fry during early stages of development were generally the most sensitive stages to chemical exposure. Rarely was reproduction or survival and growth of second generation fry reduced at exposure levels lower than those

that reduced survival or growth of the first generation fry. The authors demonstrated that for the great majority of toxicants, the quicker and more economical egg and fry tests yielded estimates of safe concentrations very similar to those derived from chronic toxicity studies.

SECTION II

MATERIALS AND METHODS

A vial containing 36.96 grams of ^{14}C -labelled 78.02, a white crystalline material, was received from the 3M Company on February 20, 1978. Based on communications with Mr. Dale Bacon of the 3M Company, it was learned that the activity of the ^{14}C -labelled 78.02 was 4.3 millicuries (mCi).

The unlabelled 78.02, also a white crystalline material, used in this study was received from the 3M Company on January 23, 1978. This material appeared to be somewhat finer in texture than the labelled 78.02 compound.

The egg and fry study was performed according to methods developed at E G & G, Bionomics (Appendix I), which closely follow those presented in the "Proposed recommended bioassay procedure for egg and fry stages of freshwater fish" (U.S. EPA, 1972).

A. Exposure System

A modified, proportional diluter similar to that described by Mount and Brungs (1967) with a dilution factor of 0.5 was used in this study. The diluent water was well water which was pumped to a concrete reservoir where it was aerated before flowing to the exposure system through PVC pipe. This water was characterized as having a total hardness and alkalinity

range as calcium carbonate (CaCO_3) of 32-35 mg/l and 28-30 mg/l, respectively (APHA, et al., 1975), a pH of 7.0-7.3 and a specific conductance of 118-140 micromhos per centimeter ($\mu\text{mhos/cm}$). The diluter delivered five nominal concentrations of ^{14}C -78.02 ranging from 2.0 to 0.13 mg/l and control water (well water), and control water containing solvent (acetone) to duplicate test aquaria. Each test aquarium measured 30.5 x 30.5 x 30.5 cm and had a standpipe drain 16 cm in height to maintain a constant test water volume of 15 l in each aquarium. The diluter delivered 0.50 l of test water to each aquarium approximately 180 times per day yielding a 90% test water replacement time of 9 hours (Sprague, 1969). To minimize the absorption of 78.02 on surfaces, all exposure system components having contact with 78.02 were constructed of acrylic material rather than glass. Ethylene dichloride was used to cement acrylic components together.

The aquaria rested in a water bath containing circulating water heated by immersion coil heaters and regulated by a mercury column thermoregulator designed to maintain the test water temperature at $25 \pm 1^\circ\text{C}$.

Due to the relatively low solubility of ^{14}C -78.02 in water, conventional toxicant delivery systems could not be utilized for this study. A metering pump (FMI Lab pump #RP-D) delivered a stock solution (19.4 mg/l, nominal ^{14}C -78.02 concentration) from a

200 l stainless steel tank to a premixing chamber on the diluter, which in turn delivered the appropriate volume of ^{14}C -78.02 solution to the mixing chamber during each diluter cycle.

A McAllister (1972) delivery device was used to deliver acetone into the solvent control aquaria at a concentration equal to the highest concentration of acetone in the 78.02 exposure aquaria ($43 \mu\text{l/l}$ acetone).

B. Egg and Fry Exposure

On June 3, 1978, the exposure of fathead minnow eggs to ^{14}C -78.02 was initiated. Eggs used were from the brood stock at the Aquatic Toxicology Laboratory of E G & G, Bionomics, Wareham, Massachusetts. Sixty eggs were randomly distributed to each of 14 egg cups and one egg cup was then suspended in each of the 14 test aquaria. Egg incubation cups were acrylic tubes 7 cm long and 5 cm in diameter with 40 mesh Nitex^R screen bottoms. An egg cup rocker arm apparatus, as described by Mount (1968), was used to gently oscillate the egg cups in the test water.

Dead eggs were removed and counted daily until hatching was completed. Percentage hatch was calculated based on the number of live fry per aquarium after hatching was completed compared to the number of eggs per aquarium (60) at the initiation of

the exposure. To initiate the 30-day fry exposure, forty fry were randomly selected from each egg cup and transferred to the respective aquaria.

Upon transfer of fry to the aquaria, the fry were fed newly hatched San Francisco Bay variety brine shrimp nauplii, ad libitum, three times daily throughout the exposure period. The aquaria were brushed and siphoned to remove excess food and fecal material three times per week. Observations on behavior and appearance of surviving fry were made daily and fry counts were made weekly. At 30 days post-hatch, the fry from each aquarium were anesthetized with MS-222 (tricaine methane-sulfonate) and percentage survival, mean total length, and average wet weight were determined. The fry were measured individually to calculate a mean and standard deviation total length while each fry group (fry from one aquarium) was wet weighed to calculate an average wet weight.

At the termination of the test, the fry from the control and the high concentration (2.0 mg/l) were preserved in Bouin's solution while the fry from the other test aquaria were frozen. Ten Bouin's solution preserved fry (5 from each replicate) from the control and the high concentration were sent to the Environmental Pathology Laboratories, Inc., Carolina, Rhode Island, July 31, 1978, for histopathological examination of a mid-line sagittal section. The remaining preserved fry and frozen fry

were sent to 3M Company, St. Paul, Minnesota, July 31, 1978.

Temperature, dissolved oxygen concentrations, and pH were monitored daily, alternating between aquaria such that each aquarium was measured once each week. Temperature was measured with a mercury thermometer, dissolved oxygen with a YSI Model #54 dissolved oxygen meter and probe, while pH was measured with an Instrumentation Laboratory Model #175 portable pH meter and probe.

C. Stock Preparation

In order to make-up the stock in the stainless steel tank, it was necessary to prepare a ^{14}C -78.02 super-stock solution first. The super-stock was formulated by quantitatively transferring the contents of the vial containing ^{14}C -78.02 and 56.16 g of non-labelled 78.02 to a 2-l volumetric flask and diluting to volume with pesticide quality acetone.

The resulting super-stock had a ^{14}C -78.02 concentration of 46.6 mg/ml and a theoretical specific activity of 102 disintegrations per minute per microgram (dpm/ μg). To determine the measured specific activity of the super-stock, three 0.050 ml aliquots were pipetted directly into glass scintillation vials containing 15 ml of Monophase^R (a xylene base counting solution with non-ionic surfactants and PPO + bis/MSB scintillators,

Packard Instrument Company), and then placed in a Model #2112 Packard Tri-Carb Liquid Scintillation Spectrometer for radiometric quantitation. The mean measured specific activity was determined to be 121 dpm/ μ g, 119% of theoretical. The stock in the stainless steel tank was prepared every 5 days by adding 83.3 ml of the super-stock solution to 200 l of well water and mixed thoroughly by stirring.

D. Sampling Schedule and Techniques

One 5.0 ml water sample was taken from each aquaria (2 per concentration) at the initiation of the test (day 0), when hatching was complete (day 3) and weekly thereafter (days 10, 17, 24 and 32) for radiometric determination of 78.02 concentrations. Samples were taken with a 5.0 ml volumetric pipet from a point approximately midway between the surface, bottom and sides of each aquarium and added to glass scintillation vials containing 15 ml of Monophase. In addition, duplicate 5.0 ml samples were taken from each 200 l stock solution immediately after preparation. The radioactivity of each sample was then quantified using liquid scintillation spectrometry.

E. Method Sensitivity and Precision

Recovery rates of the liquid scintillation spectrometer were determined prior to analyzing each set of samples by counting the activity of a standard reference material (New England

Nuclear Corporation) and comparing the measured value to the known theoretical value of the standard. Recovery rates were determined to be 99-101% and experimental data were not adjusted for percentage recovery.

Counting efficiencies of all experimental samples were determined according to the channel ratio method described by Kobayashi and Maudsley (1974). The counting efficiency for each water sample was determined by comparing the sample channel ratio to a series of quenched standards prepared monthly at E G & G, Bionomics. The standards were made by adding 15 ml of Monophase, increasing volumes (0.25 ml) of nitromethane (chemical quenching agent) and 25 μ l of a ^{14}C -toluene standard (New England Nuclear Corporation) to a series of scintillation vials containing 5 ml of well water. The measured activities of these samples reflect the change in the ratio of the sample count rate as it is altered by quenching. Days on which experimental samples were analyzed, the quenched standards were also quantitated and the data generated from the standards used to construct a channel ratio versus counting efficiency curve. From this curve, the counting efficiencies of all experimental samples were determined by calculating the sample channel ratio and interpolating the corresponding counting efficiency.

Background levels of radiation for water (35 dpm/sample) were

determined by analyzing control water samples during the study. All samples were counted for a minimum of 100 minutes or until 5,000 counts were generated. Using these criteria and the calculations described in "Standard Methods for the Examination of Water and Wastewater" (1975), it was determined at the 95% confidence level that a minimum detectable activity (minimum net cpm) above the mean background level for all samples of 20 cpm had a 7.9% counting error associated with this measurement. This percentage was the maximum accepted counting error associated with the minimum detectable limit. The percentage counting error for each sample was dependent on the net cpm of that sample and decreased as the sample activity increased.

The calculations used in determining the concentration of ^{14}C -78.02 in each water sample were as follows:

- i. gross cpm = gross counts/unit time (minute)
- ii. counting efficiency (E) = from channel ratio method
- iii. total disintegrations per minute (dpm) = gross cpm/E
- iv. net disintegrations per minute (dpm) = (total dpm) - (background dpm)
- v. total ^{14}C -78.02 ($\mu\text{g}/\text{ml}$) =
$$\frac{\text{net dpm in sample}}{\text{specific activity of the } ^{14}\text{C-78.02 x sample size}}$$

(dpm/ μg)
(ml)

F. Statistics

Means of measured biological parameters from duplicate aquaria were subjected to analysis of variance (Steel and Torrie, 1960, completely randomized block design, $P=0.05$). Data for percentage survival and percentage hatch were transformed to $\arcsin \sqrt{\text{percentage}}$ prior to analysis. When treatment effects were indicated, the means of these parameters were compared to the control means using Dunnett's procedure (Steel and Torrie, 1960). When the treatment mean was significantly different from the control mean ($P=0.05$), that treatment was considered to be an effect level.

SECTION III

RESULTS

Water quality parameters measured during the egg and fry exposure exhibited little variation between test chambers with some variation between test days. Mean and standard deviation measured dissolved oxygen concentrations for all test aquaria was 8.6 ± 0.4 mg/l and ranged from 8.0-9.9 mg/l. Mean and standard deviation temperature was $25 \pm 1^{\circ}\text{C}$ and ranged from 22-26 $^{\circ}\text{C}$. The pH ranged from 6.6-7.3, but was normally above 7.0.

The results of the radiometric analyses of weekly water samples are presented in Table 1. The mean measured ^{14}C -78.02 concentrations ranged from 90 to 112% of the nominal concentrations.

The biological data generated during this study (Table 2) indicate no adverse effects due to ^{14}C -78.02 exposure on percentage hatch of eggs or on mean length and average wet weight of fry at 30 days post-hatch. Percentage survival of 30 day old fry exposed to 1.9 mg/l measured ^{14}C -78.02 was 42%, significantly less than control fry. Mortality at this concentration was first observed after 23 days post-hatch, and continued through the remainder of the test. A majority of the fish in the 1.9 mg/l concentration were observed to be exhibiting stress behavior, erratic swimming and darkened coloration during the last 2 days of the test. A similar syndrome was observed among a few fish in the A replicate of the

1.0 mg/l concentration the last two days of the test. This occurrence suggests that the toxic action of 78.02 is cumulative and would increase with a longer exposure period. A longer fry exposure period would probably be more accurate in estimating the potential long-term hazard of ^{14}C -78.02 to fathead minnows.

Based on the reduced survival of fry at 30 days, the MTC for fathead minnow and ^{14}C -78.02 is estimated to be >1.0 mg/l and <1.9 mg/l.

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Table 1 -- Mean and standard deviation (S.D.), and range of measured concentrations of ^{14}C -78.02 during exposure of eggs and fry of fathead minnow (Pimephales promelas).

Nominal concentration (mg/l)	Measured concentration (mg/l)	
	Mean and standard deviation	Range
19.4 (stock)	19.7 (0.50)	18.7-20.5
2.0	1.9 (0.3)	1.5-2.3
1.0	1.0 (0.2)	0.7-1.2
0.50	0.45 (0.08)	0.34-0.57
0.25	0.28 (0.12)	0.17-0.49
0.13	0.12 (0.02)	0.09-0.17
control	<0.006	-
solvent control	<0.006	-

Table 2 -- Percentage hatch, percentage survival, mean and standard deviation (S.D.) total length, and average wet weight of fathead minnow fry (Pimephales promelas) during exposure to ¹⁴C-78.02.

Mean measured concentration (mg/l)	Replicate	Hatch (%)	30 Days Post-Hatch		
			Survival (%)	Total length (mm)	Wet weight (mg)
1.9	A	93	42 ^a	21(2) ^b	75
	B	97	42 ^a	20(2)	69
1.0	A	95	82	20(3)	63
	B	98	90	20(2)	62
0.45	A	95	92	21(2)	69
	B	98	88	20(2)	63
0.28	A	93	98	21(3)	66
	B	98	90	21(2)	62
0.12	A	98	95	20(2)	67
	B	97	95	20(3)	59
control	A	98	98	19(3)	54
	B	98	88	21(3)	69
solvent control	A	90	100	21(1)	67
	B	88	100	21(2)	62

^a

Significantly reduced at P=0.05.

^b

Mean and (standard deviation).

PROCEDURES FOR CRITICAL LIFE STAGE
TOXICITY TESTS WITH FRESHWATER FISHES

This describes standard toxicity testing procedures for egg and fry stages of freshwater fishes followed at the Aquatic Toxicology Laboratory of E G & G, Bionomics, Wareham, Massachusetts. This procedure closely adheres to the Proposed Recommended Bioassay Procedure for Egg and Fry Stages of Freshwater Fish (EPA, 1972).

A. Physical System

1. Diluter: A proportional diluter (Mount and Brungs, 1967) with a dilution factor of 0.5 is employed for egg and fry exposures. A check is made of diluter function by daily observations. Five toxicant concentrations, a control, and if necessary, a solvent control, are utilized in each test.
2. Toxicant mixing: A container to promote mixing of toxicant bearing solution and diluent water is used between diluter and aquaria for each concentration. Separate delivery tubes are run from this container to each duplicate tank. Calibrations are performed before every test to insure that the correct proportion of toxicant solution and diluent water is delivered to each duplicate tank. Toxicant concentrations are monitored in each duplicate aquarium.
3. Tank: Each duplicate aquarium is constructed of glass and silicone adhesive and measures 39 x 20 x 25 cm. Water depth is maintained by a constant level glass drain tube 19.5 cm from the bottom of each test aquarium. The total test solution volume in each aquarium is thus maintained at 15 l.
4. Flow rate: Five-hundred-ml of test solution are delivered to each duplicate aquarium at a rate of 6-10 tank volumes per 24 hours. This is sufficient to maintain a dissolved oxygen concentration >60% of saturation.
5. Cleaning: All aquaria are brushed and siphoned at least twice weekly.
6. Egg Cup: Egg incubation cups are made from 5 cm O.D. round glass jars with the bottoms cut off and replaced with stainless steel or Nitex^R screen (40 mesh per inch). Cups are oscillated in the test water by means of a rocker arm apparatus driven by a 2 RPM electric motor (Mount, 1968).

7. Light: When necessary for egg and fry survival (e.g., salmonids), the aquaria are shielded from all sources of light.
8. Temperature: Temperatures are controlled so as not to deviate from the specified test temperature by more than 1°C throughout the entire test period.
9. Construction materials: Construction materials which contact the test water are chosen which do not either leach or sorb significant amounts of substances from the water. Glass, silicone adhesive, Nitex^R, Tygon^R, silicone stoppers and unplasticized polyethylene are the construction materials used.
10. Water: A 125 meter deep bedrock well is the source of the diluent water. This water is pumped to a concrete holding tank where it receives extensive aeration and is delivered through aged PVC pipe to the exposure system.

B. Biological System

1. Beginning test: The exposures are initiated as soon as possible after the eggs are fertilized, and the stage of embryo development is recorded. Depending upon availability of eggs, 35 to 50 eggs are randomly distributed to each of two egg cups or 60 eggs are placed in one egg cup per duplicate aquarium. Eggs are exposed for a minimum of 1/2 the expected egg incubation period. Egg mortality in each egg cup is recorded daily. If deemed necessary, eggs will be treated with an appropriate fungicide during incubation.
2. Fry exposure: If handling of eggs permits, a daily record is kept when hatching commences of the number of eggs hatched, the number of dead fry, and the number of deformed fry in each egg cup. After complete hatch, 40 fry are randomly selected from the egg cup or cups and transferred to each aquarium. The fry are exposed to the test solution for a minimum of 30 days post-hatch. This period may be extended if the data warrants a longer investigation. The number of surviving fry is recorded twice weekly. At the end of the fry exposure period, percentage survival, individual mean total length, mean wet weight and deformities are recorded for each fry group.
3. Necessary data: Data that will be reported for

each duplicate in the egg and fry exposure are:
a) percentage hatch (number of fry surviving after hatching is complete/number of eggs incubated),
b) percentage fry survival at 30 days post-hatch,
c) growth (mean total length and weight at 30 days),
and d) deformities.

4. Food: Unless otherwise deemed necessary, fish are fed live brine shrimp nauplii twice per day ad libitum supplemented with dry pelleted food when the fish have reached a sufficient size.
5. Disease: Disease outbreaks are handled according to their nature. When treatment is deemed necessary, all aquaria will receive the same treatment.
6. Special examinations: If required, extra fish and eggs are preserved for possible future physiological, biochemical, and histological investigations which may indicate certain toxicant related effects.

C. Chemical System

1. Preparing a stock solution: Stock solutions are prepared by dissolving the toxicant in water or in an organic solvent if insoluble in water. The amount of solvent (reagent-grade or better) is kept at a minimum. If solvent is used, a solvent control is also established. The concentration of solvent in the solvent control is equal to the highest solvent concentration found in any exposure aquarium.
2. Measurement of toxicant concentrations: The concentration of toxicant is measured in each duplicate aquarium at each toxicant concentration at least once per week. Water samples are taken at a point approximately midway between the water surface, bottom and sides of each aquarium. Water samples are either extracted immediately after sampling or appropriately preserved until extractions or analyses can be performed.
3. Measurement of other variables: Temperature and dissolved oxygen are measured in aquaria daily on an alternating basis, such that each aquarium is analyzed once each week. The pH is measured weekly in the high and low test concentration and each control, alternating between replicate tanks from week to week. Total hardness is measured in the high and low concentration and control weekly. If any of these parameters are affected by the toxicant, additional

analyses are performed to more closely monitor that parameter.

4. Residue analysis: When deemed necessary, exposed fish and eggs are analyzed for toxicant residues.
5. Methods: Methods described in Methods for Chemical Analysis of Water and Wastes (EPA, 1971) are used unless other more efficient methods can provide more accurate information. Reference samples are analyzed periodically for each analytical method.

D. Statistics

1. Duplicates: True duplicates are used for each level of the toxicant being tested (i.e., no water connections between duplicate aquaria).
2. Distribution of test concentrations: The toxicant concentrations are assigned to aquaria by stratified random assignment.
3. Analysis of variance/Dunnett's

E. Miscellaneous

1. Additional information: All routine bioassay flow-through methods not covered in this procedure (e.g., physical and chemical determinations, handling of fish) closely followed those described in Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1975).
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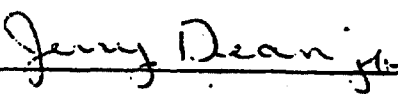
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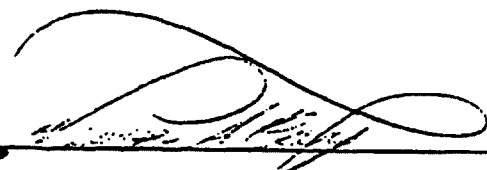
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ANWelter
ANW/cen

*Correct title is "Evaluation of the
Bioconcentration Potential of FM-3422"*

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